

Polymorphism Identification of Pit1 Gene in Indonesian Buffaloes (*Bubalus bubalis*) and Holstein-Friesian Cows

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ABSTRACT

Pit-1 gene has been identified as the pituitary specific transcription factor that regulates the expression of the growth hormone (GH) and prolactin (PRL) genes in the anterior pituitary. The use of polymorphic markers in breeding programmes could make selection more accurate and efficient. A total of 320 Indonesian buffaloes from four populations and 45 FH cows from nine populations were genotyped for polymorphism of Pit1|Hinf1 gene by the polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) methods. The gene polymorphism was detected only in FH cattle. The frequency of AA, AB, and BB genotype was 2%, 44%, and 53% respectively. The frequency of A allele was 25% and B allele was 75%, but no polymorphism was detected in 320 Indonesia buffaloes.

Key words: buffalo, Pit1 gene, PCR RFLP

INTRODUCTION

Indonesian buffaloes (*Bubalus bubalis*) is one of the important large ruminants to be improved due to its desirable characters to adapt to low quality vegetation and to survive against tropical parasites. A various number of essential functions of buffalo coming from a commonly traditional production system are basically for sources of meat, milk draft power, manure, social and culture attributes giving a significant contribution to the national livestock production.

In an effort to maintain, explore and develop the potential genetic of Indonesian buffaloes, identification of the performance and genetic parameters is needed, primarily related to important economic trait. Origin of information (gene flow), the characteristics of livestock (production traits and reproduction) and characteristics of population (diversity, the status of the population) of local buffalo yet well known. Though the information is important for the development and improvement of genetic quality of local buffalo as part of the development of national buffalo, information genetics of Indonesian buffaloes is very low. This might partly be due to the lower performance of buffaloes with respect to economic important traits when compared to cattle, limiting the interests in buffalo genome analysis in the developed

countries (Kierstein *et al.*, 2004). However, the domestic buffalo holds a great economic potential in the developing countries. Buffalo is admired as multipurpose animal for dairy, meat, and drought. The stock of domestic water buffalo is estimated to be around 130 million, which is around 1/9 of the total worldwide cattle population (FAO & IINEP, 2000).

Growth and carcass traits, which are under the control of multiple genes, are economically important traits in livestock. Selection of animals with higher growth rate and better carcass composition is of great significance to breeders and consumers. Current technologies likely enable animal breeder to improve on the accuracy and efficiency of traditional selection methods by applying genetic markers through marker-assisted selection. Pituitary-specific transcription factor responsible for pituitary development and hormone expression in mammals. Pit1, which is also termed growth hormone factor I (GHFI), is a pituitary-specific transcription factor that is responsible for pituitary development and hormone expression in mammals. Pit1 is a member of the POU domain containing proteins, which is a group of transcriptional regulators that have a critical role in differentiation and proliferation of cells (Zhao, 2002). The inhibition of Pit1 synthesis has been associated with marked decrease of both GH and PRL expression and proliferation of somatotrophic and lactotrophic cell lines (Castrillo *et al.*, 1991).

Bovine Pit1 is a 129 amino acid protein and member of DNA-binding POU family of homeo domain

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transcription factor. This has been sublocalized to the centromeric region of bovine chromosome 1, located midway between TGLA57 and RM95 (Moody *et al.*, 1995). The RFLP within bovine Pit1 gene with respect to Hinf I restriction enzyme (Pit1/Hinf I) was first identified by Woollard *et al.* (1994). Several workers since then have investigated its association with growth, body weights, carcass traits, lactating performance (Renaville *et al.*, 1997).

Pit1 polymorphism already reported in bovine, sheep, pig and goat. Moody *et al.* (1995) identified in bovines the A and B alleles of the Pit1 gene polymorphism, which is an exon 6, A to G silent mutation of the bovine Pit1 gene (Curi *et al.*, 2006) which probably is not direct responsible for phenotypic variation, although relationships have been observed between POUFI polymorphisms and body weight and somatic measures (Renaville *et al.*, 1997; Dybus *et al.*, 2004), weaning and yearling weight.

In goat, Pit1 gene locates in chromosomes 1q21-22 and is separated into six exons containing a POU domain and homeodomain (Woollard *et al.*, 2000). Lan *et al.* (2007) identified silent allele at goat Pit1 gene and association with production traits. The result showed Pit1 gene had a positive effect on milk performance and early weight trait. In 2009, Lan *et al.* (2009) identified twelve novel SNPs of the goat Pit1 gene and their associations with cashmere traits.

Sumantri *et al.* (2009) identified polymorphism of Pituitary specific transcription factor I (Pit1) gene in Indonesian local sheep. These result indicated Pit1 genotype had no significant effect on dam body weight and milk production. Yu *et al.* (1995), reported association of Pit1 polymorphisms with growth and carcass traits in pigs. These results suggest that Pit1 or a closely linked gene to Pit1 may be important in birth weight and carcass fat traits in swine.

Previous studied observed in Iranian river buffalo, indicated low variation of these genes in buffalo (Javanmard *et al.*, 2005). Renaville *et al.* (1997) showed that there is a high variation of Pit1 gene in FH cows and the A allele (especially the AB genotype) may have an effect on milk yield and its components. Therefore, the information genetic variation in pituitary specific transcription factors I (Pit1) gene is necessary observe in Indonesian buffaloes and Indonesian Friesian Holstein cows. Therefore, the purpose of this study was to analyze the genetic variability of the Pit1 gene in population of the Indonesian buffaloes and FH cows by using PCR-RFLP.

MATERIALS AND METHODS

Sample and Bleeding Location

A total of 365 (buffaloes (n= 320) and Friesian Holsteins (n=45)) were used in this study. Whole blood buffaloes were collected from four populations: Central Java (n=75), West Nusa Tenggara (n=103), North Sumatra (n=65), and Banten (n=77). In order to compare the polymorphism of pituitary specific transcription factor I (Pit-1) gene in Indonesian buffaloes, with 45 blood samples of FH cows from 9 populations: Ngantang (n=5), Boyolali (n=5), Livestock Embryo Transfer Center (n=5), Koperasi Persusuan Bandung Utara (KPSBU) (n=5), Institute for Animal Research and Technology (n=5), Bali (n=5), Kunak (n=5), Singosari (n=5), and Pasir Kemis (n=5) were analyzed. Blood samples were collected from the jugular vein using vacuum tubes containing with heparin. Blood samples were stored in absolute alcohol.

DNA-Extraction

The genomic DNA was isolated using proteinase K digestion followed by Sambrook *et al.* (1989). Blood samples were washed with destilated water and centrifuged at 8000 rpm for 5 min. The supernatant was discarded. In order to digest protein in the suspension, 400 µl lysis buffer containing proteinase K 10 µl, SDS 40 µl, and 1xSTE 350 µl and the samples were incubated at 55 °C for two hour. An equal volume of phenol-chloroform (1:1 v/v) was added and the two phases were mixed until emulsion was formed. The two phases were separated by centrifugation at 12000 rpm for 5 min. The aqueous supernatant solution was collected in fresh sterilized tubes. Phenol-chloroform extraction was repeated. The DNA was washed with 70% ethanol and dried. Finally the DNA was dissolved with elution buffer.

PCR Condition

The sequences of the forward and reverse primer for amplification of the Pit1 gene was designed based on Javanmard *et al.* (2005) No acc Y15995 and AM490263 (Table 1). The polymerase chain reaction for the Pit1 gene was performed in a 25 µl reaction mixture, containing 1 µl MgCl₂, 0.5 µl of each dNTPS, 1 µl of each primer, 10x buffer, 1 U *taq polymerase*, 2 µl genomic DNA template and 17.9 µl destilated water. The reaction mixture was placed in a PCR machine with conditions included: an initial denaturation step at 95 °C for 2 min followed by 30 cycles of 95 °C for 45 sec, 60 °C for 1 min, and 72 °C for 1 min and final extension at 72 °C for 3 min.

Table 1. Forward and reverse primers for the amplification of the Pit-1gene

Primer pair name	Primer sequence	Product size	Amplified region
Pit1 F	5'-GAGCCTACATGAGACAAGCATC-3'	611bp	Exon 6
Pit1 R	5'-AAATGTACAAATGTGCCTTCTGA-3'		

Genotyping by PCR-RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism)

Five µl PCR product of Pit1 gene were digested with restriction enzyme HinfI at 37 °C for at least 14 h. The digested fragments separated by 12% (w/v) polyacrilamid gel using a 100 base pair (bp) molecular weight standard to calculate the size of the amplified and digested fragments were visualized by silver staining and exposed to ultraviolet light. The genotypes of the individual cattle were determined for each polymorphism by analyzing the size of the fragments reported as base pairs.

Statistical Analysis

The allele and genotype frequencies were estimated by direct counting according to Nei procedure (Nei & Kumar, 2000).

$$\chi_i = \left(2n_{ii} + \sum_{j \neq i} n_{ij} \right) / (2N)$$

Where:

χ_i : gene frequency of Pit1 genotype-i

n_{ii} : number of sample with Pit1 genotype ii

n_{ij} : number of sample with Pit1 genotype ij

N : total sample population

RESULTS AND DISCUSSION

Pit1 gene fragment was successfully amplified using PCR (polymerase chain reaction) technique for all samples. The amplification of the Pit1 gene fragment resulted in a single product of 611 bp (Figure 1). Annealing temperature used for amplification of this fragment was 60 °C. This temperature was same with annealing temperature being used by Javanmard *et al.* (2005).

Polymorphic of exon 6 Pit1 gene was already described by Javanmard *et al.* (2005) in Iranian buffalo. Sequence analysis of HinfI site in the Pit1 gene revealed a mutation at position 1256 that was a G to A (Figure 2). In cattle, polymorphic in intron 5 and exon 6 in Nanyang cattle was described by Xue *et al.* (2006).

Genetic Polymorphism of the Pit1 Gene in Indonesian Buffaloes

PCR-RFLP (restriction fragment length polymorphism) using HinfI restriction enzyme was used to genotype the Indonesian buffaloes. In homozygous animals either a unique band (611 bp, AA variants) or two bands (367 and 244 bp, BB variant). Heterozygous animal gave a three band (611, 367, and 244 bp). No polymorphism was detected in exon 6 Pit1 | HinfI in four populations of Indonesian buffaloes (Banten, North Sumatera, Central Java, Nusa Tenggara Barat (NTB)). Results on Pit1 polymorphisms by HinfI enzyme show just one alleles (B) and all of the genotypes is BB (Figure 3).

In Iranian buffalo (Javanmard *et al.*, 2005), the amplification of the Pit1 gene fragment resulted in a

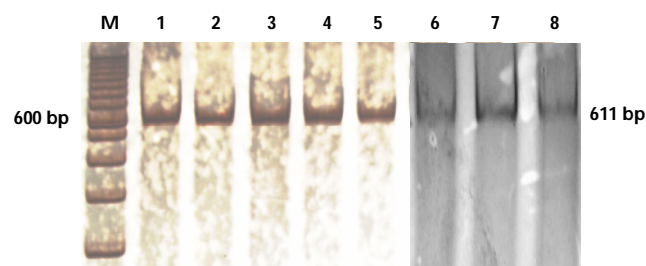


Figure 1. Polymerase chain reaction (PCR) product of Pit1 gene. M: marker; 1-5: PCR products of the Pit1 gene in buffalo; 6-8 PCR product of Pit1 gene in FH cattle.

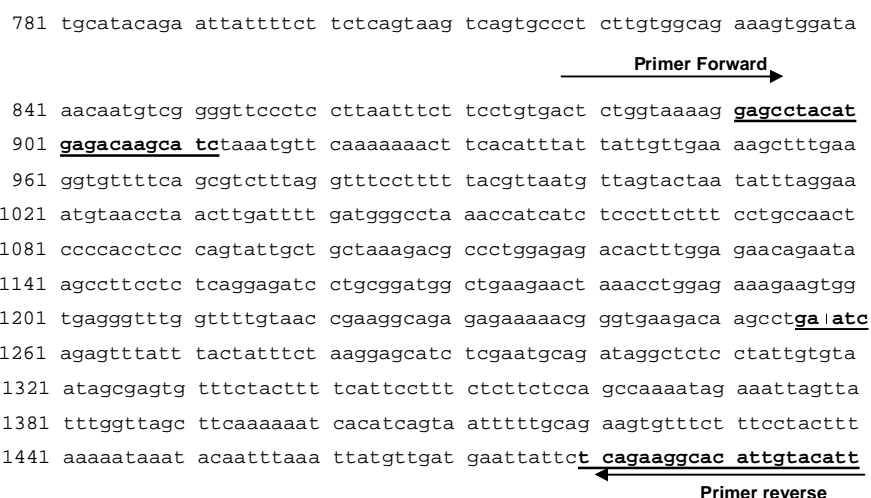


Figure 2. The pattern corresponding to the sequence Pit1 gene in cattle (Gene Bank accession Y15995 dan AM490263). Restriction site mutation at position 1256 (G to A).

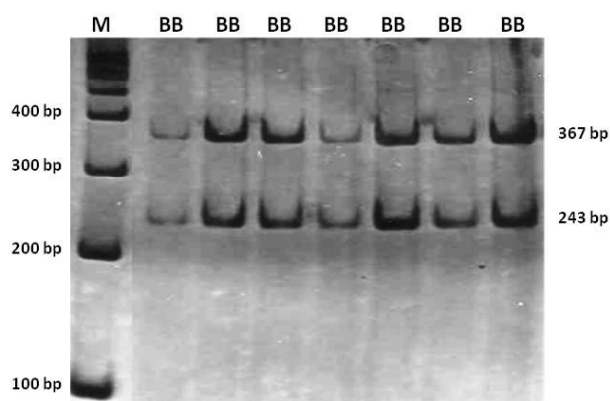


Figure 3. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) electrophoretic pattern of exon 6 of Pit1 gene of the Indonesian local buffalo in PAGE 12% showing genotype BB.

single product of 600 bp. In homozygous animal either a unique band (600 bp, AA variants) or two bands (357 bp and 243 bp, BB variant). Heterozygous animal gave three band (600, 357 and 243 band).

Genetic Polymorphism of the Pit1 Gene in Indonesian Friesian Holstein Cows

Blood samples from nine populations in Indonesian FH cows were successfully identified by using PCR-RFLP method. HinfI restriction enzyme was used to genotype the Indonesian FH cows. Restriction pattern of Pit1 HinfI gene have three genotypes of AA, AB, and BB upon digestion of the Pit1 HinfI. AA variants showed single band (611 bp), BB variants showed two band (357 and 243 bp), and AB variants showed three bands (611 bp, 367 bp, and 243 bp) (Figure 4).

Similar findings were obtained by many workers who detect the fragments after digestion (Woollard, 1994; Zwierzchowski, 2002; Javanmard *et al.*, 2005). Pit1 polymorphism in bovine and buffalo was detected by Zhao *et al.* (2004) used SSCP method. In human, Pit1

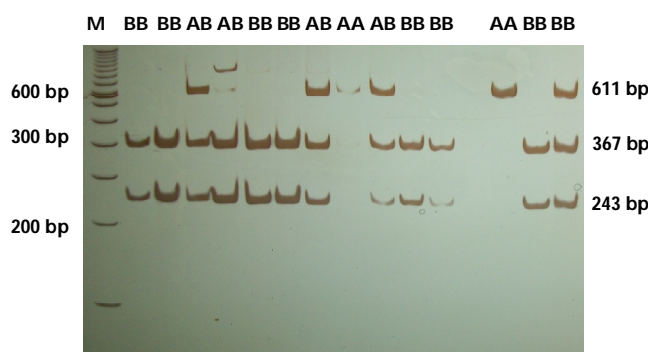


Figure 4. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) electrophoretic pattern of exon 6 of Pit1 gene of the Indonesian FH cattle in PAGE 12%.

polymorphism was detected by Kishimoto *et al.* (2002). Sequence analysis of the polymorphic HinfI site revealed a mutation at position 1256. The mutation was a G to A transition (Figure 5).

Allel A : 5'---- GACAAGCCTAAATCAGAGTTTAT----- 3'
Allel B : 5 ---- GACAAGCCTGAATCAGAGTTTAT----- 3'

Figure 5. Sequence analysis of the polymorphic HinfI site revealed a mutation at position 1256. The mutation was a G to A transition.

Genotype and Gene Frequencies of Pit1|HinfI in Indonesian Buffaloes

Table 2 showed there is no variation genotype in Indonesian buffaloes. Considering the 320 buffalo analyzed, the overall genotype frequencies was BB (100%). The Pit1 B allele frequency was observed to be the highest (100%) in the studied population (Table 1). This result was different to that reported gene frequencies of Pit1 variant in Iranian buffalo (Javanmard *et al.*, 2005). In Iranian buffalo genotype frequencies were 0.567 for AA, 0.4 for AB, and 0.3 For BB. In which, gene frequencies for A allele was 0.766 and B allele was 0.3578.

In Iranian buffalo, gene frequency of A allele was higher than that of B allele. But in Indonesia, gene frequency of B allele was higher than A allele. Different result might be caused by different species of buffalo. Javanmard *et al.* (2005) identified polymorphism of Pit1|HinfI in river buffalo, but in this research, identified polymorphism of Pit1|HinfI in swamp buffalo.

Renaville *et al.* (1997) reported the association of A allele at Pit1|HinfI locus with superior milk and protein yields and inferior for fat percentage in dairy cattle. Similarly Zwierzchowski *et al.* (2002) showed that the allele A of the Pit1 locus had positive effect on milk production traits. In beef cattle, Zhao *et al.* (2004) reported that Pit1|HinfI polymorphism appears to effect growth traits in Angus cattle and may be a candidate gene for Marked Assisted Selection (MAS).

Genotype and Gene Frequencies of Pit1|HinfI in Indonesian FH cows

Genotype frequencies of 45 Friesian-Holstein cows were 0.02 for AA, 0.44 for AB, and 0.53 for BB. Gene frequencies of A allele and B were 0.25 and 0.75 respectively (Table 4). This results were higher than those the frequencies obtained by Zwierzchowski *et al.* (2001) for different breeds of Bos Taurus, Angus (0.55), Holstein (0.74), Brahman (0.75), Polish (0.75) and Gry cattle (0.05) but lower than Hereford (0.79), and Glbvieh (0.9) breed (Zwierzchowski *et al.*, 2002).

This fact is almost the same with the previous observations of Renaville *et al.* (1993). The AA pattern was less frequent than the AB or BB patterns. The significant superiority of the Pit1 A allele over the B allele was observed for milk yield, protein yield, body depth, angularity, and rearleg set (Jawasreh *et al.*, 2009).

Lan *et al.* (2007) identified the silent allele at the goat Pit1 locus. Genomic DNA samples were obtained from

Tabel 2. Genotype and gene frequencies of the Pit1|Hinf1 in the Indonesian buffaloes

Population	N	Genotype frequencies			Allele frequencies	
		AA	AB	BB	A	B
NTB	103	0	0	103(1.00)	0	1
Central Java	75	0	0	75(1.00)	0	1
North Sumatera	65	0	0	65(1.00)	0	1
Sum	243	0	0	243(1.00)	0	1

Table 3. Genotype and gene frequencies of the Pit1|Hinf1 in the Friesian-Holstein cows

Location	N	Genotype frequencies			Allele frequencies	
		AA	AB	BB	A	B
Ngantang	5	0 (0.00)	1 (0.20)	4 (0.80)	0.1	0.9
Boyolali	5	0 (0.00)	1 (0.20)	4 (0.80)	0.1	0.9
BET	5	0 (0.00)	2 (0.40)	3 (0.60)	0.2	0.8
KPSBU	5	0 (0.00)	2 (0.40)	3 (0.60)	0.2	0.8
BPPT	5	0 (0.00)	3 (0.60)	2 (0.40)	0.3	0.7
Bali	5	0 (0.00)	3 (0.60)	2 (0.40)	0.3	0.7
Kunak	5	1 (0.20)	1 (0.20)	3 (0.60)	0.3	0.7
Singosari	5	0 (0.00)	4 (0.80)	1 (0.20)	0.4	0.6
Pasir Kemis	5	0 (0.00)	3 (0.60)	2 (0.40)	0.3	0.7
Sum	45	1 (0.02)	20 (0.44)	24 (0.53)	0.25	0.75

801 individuals belonging to nine native Bp breeds. PCR product of Pit1 gene were digested with AluI restriction enzyme. There were three genotypes, CC, TC and TT. Lan *et al.* (2007) reported relationships between AluI PCR-RFLPs and eight production traits of 663 samples were done. Significant association of different genotypes with milk yield ($P < 0.05$) and birth weight ($P < 0.05$) implied that Pit1 gene had positive effect on milk performance and early weight. While goat Pit1 gene did not significant effect on her traits.

Genetic variability (Tables 2 and 3) results in both cattle breeds for the two studied genes indicated the low variation. The high prevalence of B allele and BB genotype suggested almost near fixation of B allele in Indonesian buffaloes. That may be indicated the specific allele in Indonesian buffaloes.

CONCLUSION

No polymorphism found in Pit1|Hinf1 locus in Indonesian buffaloes. The overall genotype frequencies for Pit1 gene was BB (100%). Therefore, Pit1|Hinf1 locus could not be used as a marker for meat production. There was high variation of Pit1 gene in Friesian Holstein cows with genotype frequencies of AA, AB, and BB were 0.02, 0.44, and 0.53 respectively. Gene frequencies of A allele and B allele in Pit1 were 0.25 and 0.75, respectively. Association between Pit1|Hinf1 locus variation with milk production for Friesian Holstein cows could be further investigated.

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